Glucagon-like Peptide-1 Stimulates Luteinizing Hormone–releasing Hormone Secretion in a Rodent Hypothalamic Neuronal Cell Line

Sarah A. Beak,* Melanie M. Heath,* Caroline J. Small,* David G.A. Morgan,* Mohammad A. Ghatei,* Amanda D. Taylor,‡ Julia C. Buckingham,‡ Stephen R. Bloom,* and David M. Smith*

**Division of Endocrinology and Metabolic Medicine, Department of Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 ONN, United Kingdom; and ‡Department of Pharmacology, Charing Cross and Westminster Medical School, London W6 8RF, United Kingdom*

Abstract

To examine the influence of the putative satiety factor (GLP-1) on the hypothalamo-pituitary-gonadal axis, we used GT_1-7 cells as a model of neuronal luteinizing hormone–releasing hormone (LHRH) release. GLP-1 caused a concentration-dependent increase in LHRH release from GT_1-7 cells. Specific, saturable GLP-1 binding sites were demonstrated on these cells. The binding of [^{125}I]GLP-1 was time-dependent and consistent with a single binding site (K_d = 0.07±0.016 nM; binding capacity = 160±11 fmol/mg protein). The specific GLP-1 receptor agonists, exendin-3 and exendin-4, also showed high affinity (K_i = 0.3±0.05 and 0.32±0.06 nM, respectively) as did the antagonist exendin-(9–39) (K_i = 0.98±0.24 nM). At concentrations that increased LHRH release, GLP-1 (0.5–10 nM) also caused an increase in intracellular cAMP in GT_1-7 cells (10 nM GLP-1: 7.66±0.4 vs. control: 0.23±0.02 nmol/mg protein; $P < 0.001$). Intracerebroventricular injection of GLP-1 at a single concentration (10 μg) produced a prompt increase in the plasma luteinizing hormone concentration in male rats (GLP-1: 1.09±0.11 *vs.* saline: 0.69±0.06 ng/ml; $P < 0.005$). GLP-1 levels in the hypothalami of 48-h–fasted male rats showed a decrease, indicating a possible association of the satiety factor with the low luteinizing hormone levels in animals with a negative energy balance. (*J. Clin. Invest.* 1998. 101:1334–1341.) Key words: receptors • rat-Wistar • peptides-pharmacology • cell line • hypothalamus

Introduction

Glucagon-like peptide-1(7–36) amide (GLP-1)[1] is processed from proglucagon in intestinal L cells (1), and is a member of the structurally related glucagon/secretin family of peptides. Furthering its well-documented roles in the periphery, more recent studies indicate a role for GLP-1 as a brain neuropeptide. The processing of proglucagon in the central nervous system (CNS; references 2–4) resembles that in the intestine (5), with GLP-1 as a major biologically active product. Immunocytochemical studies have demonstrated the presence of GLP-1 immunoreactive cell bodies in the nucleus of the solitary tract and the medullary reticular nucleus in the rat brain (6). The peptide has also been identified in nerve fibers in medial hypothalamic structures including the hypothalamic paraventricular nucleus and the periventricular strata (4, 6, 7). The presence of GLP-1 in the synaptosome fraction of the hypothalamus, its calcium-dependent release from hypothalamic tissue slices by potassium stimulation (4), and the existence of specific receptors for GLP-1 in the CNS (8) support a putative neurotransmitter or neuromodulatory role for this peptide.

The GLP-1 receptor belongs to a distinct subclass of the G-protein–coupled receptor family that includes receptors for glucagon, secretin, vasoactive intestinal peptide, growth hormone–releasing hormone, parathyroid hormone, and calcitonin (for review see reference 9). Specific CNS GLP-1 receptors have been demonstrated in this laboratory and elsewhere, in hypothalamic and thalamic nuclei, in the brain stem, and in the pituitary gland (7, 10–12). Moreover, after elucidating the GLP-1 receptor nucleotide sequence (13), in situ hybridization studies demonstrated sites of GLP-1 receptor synthesis in both neurons and glia of the hypothalamus, thalamus, hippocampus, primary olfactory cortex, choroid plexus, and pituitary gland (14). More detailed studies of the distribution of GLP-1 receptor mRNA in the hypothalamus revealed dense accumulation of labeled cells in the supraoptic, paraventricular, and arcuate nuclei. Additional labeled cells were also detected in medial and lateral preoptic areas, the periventricular nucleus, the ventral division of the bed of stria terminalis, the lateral hypothalamus, and the dorsomedial nucleus (15).

GLP-1 immunoreactivity is widely distributed and colocalized with specific GLP-1 binding sites in regions of the CNS associated with the control of various endocrine and behavioral responses (16), evidence that supports a role for GLP-1 as a modulator of neuroendocrine and/or autonomic outflow. We and others have demonstrated that GLP-1 inhibits food (10, 17, 18) and water (18) intake when given intracerebroventricularly to rats. In addition, we have identified a potential role for GLP-1 in the regulation of thyrotropin release, which appears to involve direct actions of the peptide on the anterior pituitary gland (19). Taking into consideration these findings, in particular its role as a known satiety factor in the CNS (10, 17, 18), it is possible that GLP-1, like several other hypothalamic neuropeptides (20, 21), acts as a metabolic signal to the reproductive system. In the present study we have primarily used the luteinizing hormone–releasing hormone (LHRH)–secret-

Address correspondence to David M. Smith, Division of Endocrinology and Metabolic Medicine, Department of Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, London W6 8RF, United Kingdom. Phone: +44-181-383-4552; FAX: +44-181-383-3142; E-mail: dsmith@rpms.ac.uk

Received for publication 12 May 1997 and accepted in revised form 12 January 1998.

1. *Abbreviations used in this paper:* aCSF, artificial cerebrospinal fluid; B_{max}, binding capacity; Bq, becquerels; CNS, central nervous system; GLP-1, glucagon-like peptide-1; LH, luteinizing hormone; LHRH, luteinizing hormone–releasing hormone.

J. Clin. Invest.

0021-9738/98/03/1334/08 $2.00
Volume 101, Number 6, March 1998, 1334–1341
http://www.jci.org

ing hypothalamic cell line GT_1-7 to examine the direct effects and mechanism of action of GLP-1 on LHRH release. We also examined the effect of intracerebroventricular injection of GLP-1 on LH release in vivo in male rats. To investigate a possible role for changes of hypothalamic GLP-1 content in the reduction of LH release observed in negative energy balance, we determined the effect of a 48-h fast and refeeding on hypothalamic GLP-1 content.

Methods

Materials. GLP-1 and the exendin peptides were synthesized using an automated peptide synthesizer (model 396 MPS; Advanced Chemtech Inc., Louisville, KY) and checked for correct molecular weight by mass spectroscopy. $Na^{125}I$ was supplied by Amersham International (Little Chalfont, United Kingdom). All tissue culture materials were supplied by Life Technologies (Paisley, United Kingdom), and all other reagents by Merck (Poole, United Kingdom) or Sigma Chemical Co. (Poole, United Kingdom).

GT_1-7 cell culture and membrane preparations. The immortalized hypothalamic LHRH-producing neurone subclone GT_1-7 cells, which are derived from transgenic mice expressing a hybrid gene composed of the 5′ flanking region of the rat *LHRH* gene linked to cDNA encoding the SV40 T antigen (22), were grown in 175-ml plastic culture flasks. They were maintained at 37°C in 6% CO_2 in DME with 4.5 g/liter glucose, 10% FBS, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Culture medium GLP-1 levels were measured by RIA (23) and were found to be below the detection limit of the assay (0.5 fmol/tube with 95% confidence). To prepare membranes, cells (10–12 confluent flasks) were recovered by scraping cells into 20 ml homogenization buffer (50 mM Hepes, pH 7.4, containing 0.25 M sucrose, 10 μg/ml soybean trypsin inhibitor, 0.5 μg/ml pepstatin, leupeptin and antipain, 0.1 mg/ml benzamidine, 0.1 mg/ml bacitracin and 30 μg/ml aprotinin). Subsequent homogenization (6 × 30 s) using an UltraTurrax homogenizer (Merck) resulted in cell breakdown, which was verified by microscopic examination. The homogenate was centrifuged at 1,000 *g* for 10 min at 4°C, and the resulting supernatant was collected and centrifuged at 100,000 *g* for 1 h at 4°C. The pellet was resuspended in ~5 ml of homogenization buffer without sucrose using a hand-held glass Teflon homogenizer (Merck). Protein concentration was measured by the Biuret method (24), and aliquoted membranes (3 mg/ml) were stored at −80°C.

Secretion experiments. GT_1-7 cells were plated on poly-L-lysine–coated 24-well plates. After growing for 2–3 d, cells were washed twice in DME with 4.5 g/liter glucose, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 0.1% BSA. The cells were then preincubated for 2 h in serum-free medium. Thereafter, the medium was discarded, and the cells were incubated in 500 μl of medium plus the appropriate test substance (GLP-1, 0.01–100 nM or 56 mM KCl) for 60 min. At the end of the incubation period, medium was removed and frozen at −20°C. Culture medium LHRH levels were measured using RIA (reagents and methods provided by Dr. H.M. Fraser, Medical Research Council Reproductive Biology Unit, Edinburgh, Scotland). Protein levels from each well were calculated using the Bio-Rad protein assay (25), and LHRH release was expressed per milligram of protein.

GLP-1–binding studies. GLP-1–binding studies were essentially carried out as previously described (19). In brief, GLP-1 was labeled with $Na^{125}I$ by the chloramine-T method, and the monoiodinated peptide was purified by high-performance liquid chromatography as described previously (19). The specific activity of the label, determined by RIA (23), was 50±4.2 becquerels (Bq)/fmol (n = 6). GT_1-7 cell membranes (50 μg) were incubated with [^{125}I]GLP-1 (750 Bq, 30 pM) for 90 min at 20°C in a final assay volume of 0.5 ml. Specific binding was calculated as the difference between the amount of [^{125}I]GLP-1 bound in the absence (total) and presence (nonspecific) of 200 nM unlabeled peptide. Dissociation was measured at 20°C and 37°C after adding 1 μM (final concentration) of unlabeled GLP-1 and 500 μM guanosine 5′-triphosphate (GTP) to membranes, and [^{125}I]GLP-1 (30 pM) in equilibrium after a 90-min incubation. Equilibrium saturation experiments were performed using 25 μg of membrane protein and label from 40–1,600 pM (500–20,000 Bq/assay) in an assay volume of 0.25 ml. In equilibrium competition experiments, the concentration of unlabeled peptide was from 0 to 200 nM. Binding data were analyzed by nonlinear regression using the Receptor Fit programme (Lundon Software, Cleveland, OH) to calculate the dissociation constant (K_d), the absolute inhibition constant (K_i), and the concentration of binding sites (B_{max}). The integrity of the label after binding was determined by 10% trichloroacetic acid precipitation. Degradation of the label was always < 10% regardless of the time of incubation (data not shown).

Determination of cAMP generation. GT_1-7 cells were plated onto poly-L-lysine–coated 24-well plates, as described above. After growing for 2–3 d, cells were again washed twice in serum-free DME and preincubated for 2 h. Thereafter, the medium was discarded, and the cells were incubated for 10 min in DME containing 2 mM 3-isobutyl-1-methyl-xanthine alone, or with GLP-1 (0.01 nM-10 nM), forskolin (10 μM, 100 μM), or GLP-1 (1 nM) plus exendin-(9–39) (1–100 nM). The cells were washed once with 500 μl of PBS and extracted in 500 μl of 75% ethanol containing 16 mM HCl, at −20°C overnight. Samples were dried by rotary evaporation, and cAMP concentrations were assessed using a cAMP radioimmunoassay kit (Du Pont, Stevenage, United Kingdom). Protein levels were calculated from representative wells on the plates using the Bio-Rad protein assay (25), and cAMP levels were expressed per milligram of protein.

Cytoplasmic Ca^{2+} measurements. Cytoplasmic calcium was measured in GT_1-7 cell suspensions. GT_1-7 cells were cultured to 80–90% confluence in plastic 175-ml flasks as described above. Cells were then loaded with fura-2 at 37°C by incubation in 5 μM fura-2-AM (Novabiochem, Nottingham, United Kingdom) for 30 min. The fura-2-AM containing extracellular medium was subsequently removed, and the cells were scraped into 20 ml of assay buffer (10 mM Hepes, pH 7.2, containing 140 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, and 6 mM glucose). Spectrofluorometric analysis of Ca^{2+} was conducted at 22°C as described previously (27) using a Shimadzu RF5001PC (Shimadzu Experimental Supplies, Kyoto, Japan), and analysis of the ratio between fluorescence was measured at 340 and 380 nm. GLP-1 (up to 5 μM) was compared with K^+ (20 mM) and histamine (100 μM) as positive controls.

Investigation of the effect of fasting on rat hypothalamic GLP-1 content. Adult male Wistar rats (200–220 g) were maintained in groups of five animals per cage under controlled temperature (21–23°C) and light (11 h of light, 13 h of darkness) with ad libitum access to chow and water. At the beginning of the study the animals were divided into three groups (n = 15). Group 1 animals were fasted for 48 h, group 2 animals were fasted for 48 h and then refed for 12 h in the dark (feeding) phase, and group 3 animals acted as control with ad libitum access to chow. All animals had ad libitum access to water. The rats were killed by decapitation, followed by immediate microdissection of whole hypothalami, which were frozen in liquid nitrogen. The tissue removed was bordered rostrally by the anterior edge of the optic chiasma, laterally by the hypothalamic fissures, and caudally by the mamillary bodies. The ventral border of the dorsal extent of the cut was at a depth of no more than 2 mm. Tissue extraction was carried out as previously described (4). In brief, each hypothalamus was placed into 500 μl of 0.5 M acetic acid at 100°C for 10 min. The extract was stored at −20°C. GLP-1 levels were measured by RIA (23). Protein concentration in the extract was calculated using the Bio-Rad protein assay (25), and GLP-1 content was expressed as fmol per mg of extracted protein.

In vivo LH studies. Adult male Wistar rats (200–220 g) were maintained as above. Rats were anaesthetized, and stainless steel guide cannulae were implanted into the third cerebral ventricle as previously described (10, 28). After a 7-d recovery period, rats failing to exhibit a drinking response to intracerebroventricular injection of

angiotensin II (150 ng in 10 μl) were excluded (< 10%). Animals displaying a positive drinking response were sham-injected before the study, and weighed and handled daily.

GLP-1 was dissolved in 0.9% saline and administered intracerebroventricularly as previously described (28). Each study involved an injection of 10 μl of GLP-1 (10 μg) or saline. After intracerebroventricular injection, rats were returned to their cages and decapitated after 5 min. Trunk blood was collected in plastic lithium heparin tubes containing 0.6 mg aprotinin. Plasma was separated by centrifugation, frozen immediately on dry ice, and stored at −20°C. Plasma LH was measured using the reagents and methods provided by the NIDDK and the National Hormone and Pituitary Program (Dr. A. Parlow, Harbor-University of California-Los Angeles Medical Center, Los Angeles, CA). A double antibody separation system was employed, which used a goat anti–rabbit solid-phase antibody (Kabi AB, Stockholm, Sweden) and 0.1% Triton-X 100. Results were calculated in terms of NIDDK standard preparation (NIDDK rat LH-RP-3).

Static incubation of whole hypothalami. The static incubation system used was a modification of the method previously described (29). Adult male Wistar rats (200–220 g) were maintained in groups of five animals per cage as above. On the day of the study, the rats were killed by stunning and decapitation, followed by immediate microdissection of whole hypothalami as described above. The tissue blocks (< 35 mg wet wt each) were transferred to individual tubes containing 1 ml artificial cerebrospinal fluid (aCSF; 20 mM $NaHCO_3$, 126 mM NaCl, 0.09 mM Na_2HPO_4, 6 mM KCl, 1.4 mM Ca_2Cl, 0.09 mM $MgSO_4$, 1 mM glucose, 0.18 mg/ml ascorbic acid, and 100 μg/ml aprotinin). The tubes were placed on a shaking platform in a water bath (maintained at 37°C in 95% O_2, 5% CO_2) and preincubated for 60 min. The hypothalami were then incubated for 30 min in 0.5 ml fresh aCSF (basal release) before being challenged with 1 μM GLP-1 in 0.5 ml fresh aCSF for 60 min. Finally, the viability of the tissue was verified by a 30-min exposure to 56 mM KCl. Isotonicity was maintained by substituting Na^+ for K^+. LHRH concentrations were measured by RIA as above and expressed per milligram wet weight tissue per h.

Statistics. Results are shown as mean values±SE. For the GT_1-7 cell cAMP, LHRH, and hypothalamic GLP-1 content assays, data were compared by repeated measures ANOVA with subsequent post hoc Tukey's tests (Systat, Evanston, IL) between control and experimental groups, with $P < 0.05$ considered to be statistically significant. For the LH and whole hypothalami LHRH assays, data were compared by unpaired and paired Student's *t* test, respectively, with $P < 0.05$ considered to be statistically significant. For binding data, analysis of one-site vs. two-site competition curves was by F-test, with two component fits considered significant at $P < 0.05$.

Results

Effect of GLP-1 on LHRH release in GT_1-7 cells. The effect of GLP-1 on LHRH release from GT_1-7 cells in static culture was examined (Fig. 1). Exposure of the cells to GLP-1 for 60 min resulted in dose-dependent stimulation of LHRH release with a significant effect of dose vs. response [$F(7,65) = 93.68$; $P < 0.0001$]. At concentrations of 0.5, 1.0, and 10 nM, GLP-1 produced pronounced LHRH responses that were significantly increased when compared with basal levels. Basal release was 325±17 fmol/mg protein, and GLP-1 (10 nM) increased this to a maximum of 242% of basal (787±45 fmol/mg protein; $P < 0.0001, n = 4$). Control cells were exposed to 56 mM KCl; K^+ stimulated LHRH release to 1467±65 fmol/mg protein ($P < 0.0001$, $n = 4$). The EC_{50} for GLP-1–stimulated LHRH release from GT_1-7 cells was calculated to be 0.28 nM.

Characterization of [^{125}I]GLP-1 binding in GT_1-7 cell membranes. [^{125}I]GLP-1 (30 pM)-specific binding to 50 μg cell membranes for 90 min at 20°C represented 87.7±1.0% ($n = 10$) of the total radioactivity bound. Specific binding was time-

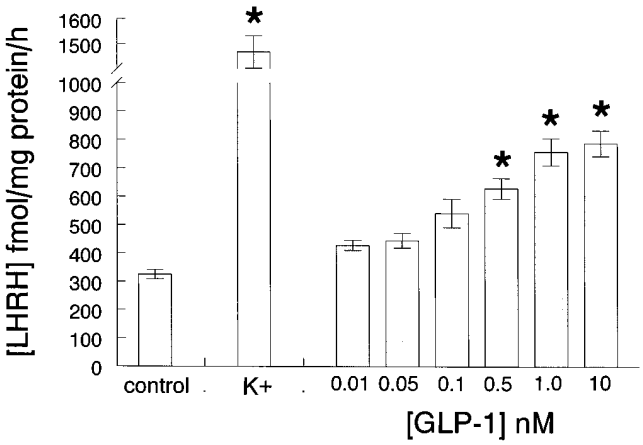

Figure 1. GLP-*1* stimulation of basal LHRH secretion from GT1-7 cells. Culture medium LHRH levels were measured by RIA after 60 min of incubation in the presence of GLP-1 (0.01–10 nM) or 56 mM KCl. Results are the mean±SE of four experiments performed in quadruplicate and expressed as fmoles of LHRH secreted per milligram of protein per h. [$F(7,65) = 93.68$, $P < 0.0001$]. (*$P < 0.0001$ vs. control).

(Fig. 2) and temperature- (data not shown) dependent. The association and dissociation time courses are shown in Fig. 2. Tracer-specific binding can be seen to reach steady-state by ~ 90 min. Very slight dissociation was observed upon addition of 1 μM unlabeled GLP-1 to membranes in equilibrium with the label (data not shown). However, on addition of both 1 μM unlabeled peptide and 500 μM GTP, dissociation was ~ 50% and 65% complete after 200 min at 20°C and 37°C, respectively, suggesting involvement of guanine nucleotides and G-protein linkage of the receptor. Dissociation was more pro-

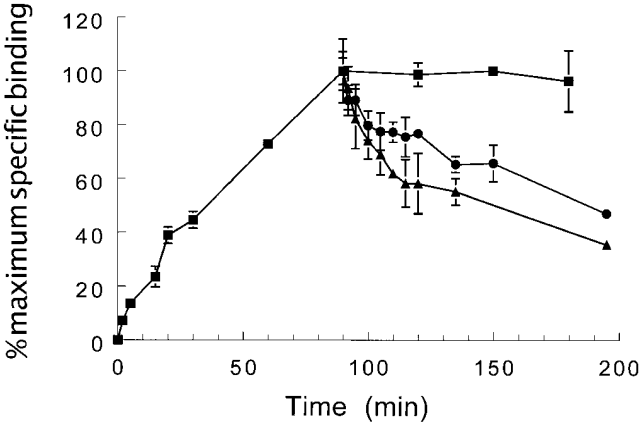

Figure 2. Kinetics of [^{125}I]GLP-1 binding in GT1-7 cells. Association (■, 20°C) and dissociation (●, 500 μM GTP at 20°C; ▲, 500 μM GTP at 37°C) of [^{125}I]GLP-1 (30 pM) in GT_1-7 cell membranes (50 μg). All points are the mean±SE of triplicate assays in three separate experiments. Dissociation of [^{125}I]GLP-1 in equilibrium was initiated at 90 min by adding unlabeled GLP-1 to a final concentration of 1 μM and 500 μM GTP at 20°C and 37°C. Bound and free were separated by rapid filtration under reduced pressure. Where no *error bars* are shown, they did not exceed the limits of the symbols.

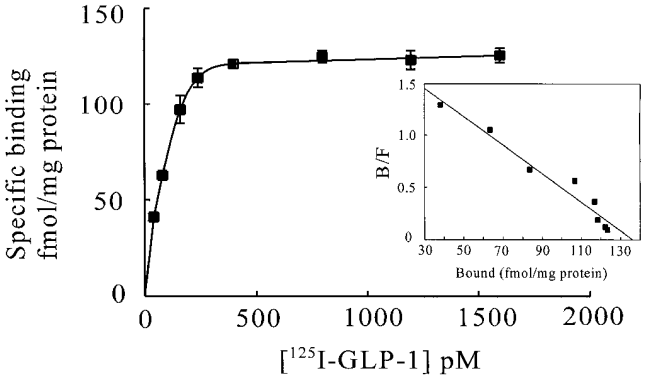

Figure 3. Equilibrium saturation analysis of [^{125}I]GLP-1 binding. The effect of increasing concentrations of [^{125}I]GLP-1 on specific binding in GT_1-7 cell membranes (25 μg) was determined. Results are expressed as the mean specific binding±SE of triplicate assays in three separate experiments. A Rosenthal plot is shown in inset, where *B* is bound ligand and *F* is free ligand. This figure is intended as a pictorial representation of the above data. Actual B_{max} values were calculated by nonlinear regression.

nounced at 37°C than 20°C, and appeared to be represented by two components, with a more rapid initial component. Specific [^{125}I]GLP-1 binding at 20°C was proportional to membrane protein up to 200 μg (data not shown). Consequently, for equilibrium competition binding studies, 50 μg membrane protein were incubated with 30pM [^{125}I]GLP-1 plus unlabeled GLP-1 for 90 min at 20°C.

Fig. 3 shows equilibrium saturation analysis that was carried out to establish saturation of binding and to determine the affinity and density of the sites. Analysis of the data indicated a single class of binding site with an estimated K_d of 0.07±0.016 nM (n = 3) and a B_{max} of 160±11 fmol/mg protein. Fig. 4 shows the results from equilibrium competition binding studies that were designed to determine the affinity and specificity of the binding of various GLP-1 receptor ligands to GT_1-7 cell membranes. Competition curves were constructed using GLP-1, the exendin peptide agonists exendin-3 and exendin-4, and the truncated peptide antagonist exendin-(9–39). Tracer-specific binding was inhibited in a concentration-dependent manner by all four unlabeled peptides. The rank order of potency was as follows: GLP-1 (K_d = 0.3±0.08 nM; n = 3) = ex-

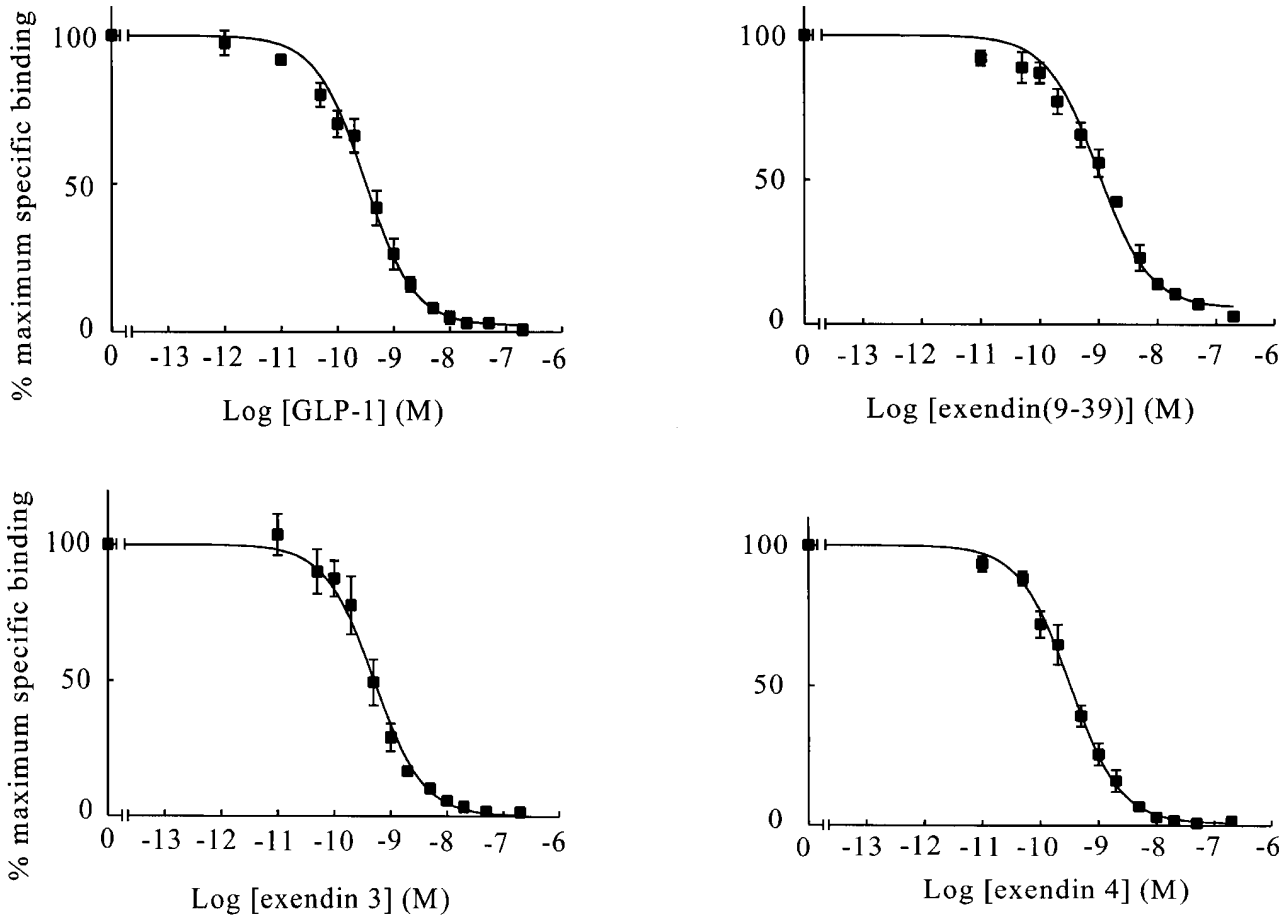

Figure 4. Equilibrium competition analysis of [^{125}I]GLP-1 binding. Competition of [^{125}I]GLP-1 (30 pM) binding in GT_1-7 cell membranes (50 μg) by GLP-1, exendin-3, exendin-(9–39) and exendin-4 was determined. Values are expressed as percentage of maximal specific binding, and are the mean of three separate experiments±SE with assays performed in triplicate. Where no *error bars* are shown, they did not exceed the limits of the symbols.

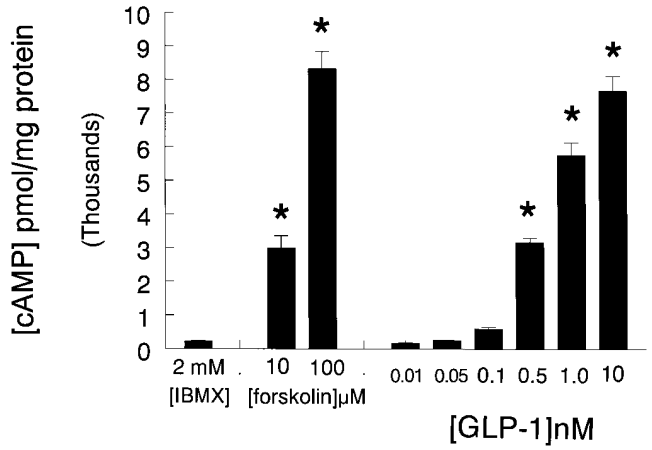

Figure 5. Effect of GLP-1 on cAMP production in GT1-7 cells. cAMP levels were determined after a 10-min incubation in medium containing 2 mM IBMX alone or together with GLP-1 (0.01–10 nM) or forskolin (10 μM, 100 μM). Results are the mean±SE of four experiments performed in quadruplicate and expressed as pmol cAMP per milligram of protein. [F(8,63) = 129.25; $P < 0.0001$]. (*$P < 0.0001$ vs. control).

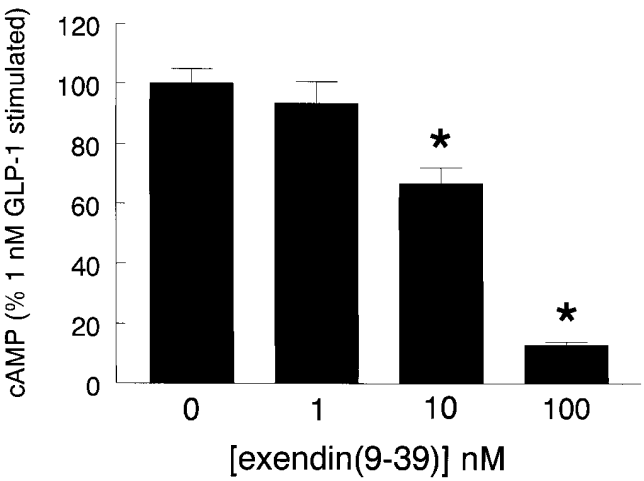

Figure 6. Exendin-(9–39) blockade of GLP-1 stimulated cAMP production in GT1-7 cells. cAMP levels were determined after a 10-min incubation in medium containing 2 mM IBMX plus 1 nM GLP-1 alone or together with increasing concentrations of exendin-(9–39). Results are the mean±SE of four experiments performed in quadruplicate and expressed as a percentage of GLP-1 induced cAMP stimulation. Inhibition is concentration-dependent [F(3,54) = 65.16, $P <$ 0.0001]. [*$P < 0.0001$ exendin-(9–39) vs. GLP-1 (1 nM)].

endin-3 (K_i = 0.3±0.05 nM; n = 3) exendin-4 (K_i = 0.32±0.06 nM; n = 3) > exendin-(9–39) (K_i = 0.98±0.24 nM; n = 3).

The structurally related peptides—glucagon, pituitary adenylate cyclase–activating polypeptide, and vasoactive intestinal peptide—and the unrelated peptides—neuropeptide Y and calcitonin gene-related peptide—in concentrations up to 1 μM were unable to inhibit [^{125}I]GLP-1 binding, thereby establishing the specificity of binding (data not shown).

Effect of GLP-1 on cAMP production and intracellular Ca^{2+} levels in GT_1-7 cells. Fig. 5 shows the effect of GLP-1 on intracellular cAMP levels. These studies were performed to determine whether the GLP-1 binding site on the GT_1-7 cell membranes was functionally coupled to the adenylyl cyclase system. Basal production of cAMP in the unstimulated cells was 0.23±0.02 nmol/mg protein (n = 4). GLP-1 stimulated cAMP production in a concentration-dependent manner with a significant effect of dose vs. response [F(8,63) = 129.35; $P <$ 0.0001]. Exposure to 0.5, 1.0, and 10 nM GLP-1 produced significant increases in cAMP in comparison to basal levels. The greatest cAMP formation obtained (with 10 nM of the peptide) was 7.76±0.4 nmol/mg protein, i.e., 3276% above the basal level ($P < 0.0001$, n = 4). Control cells were exposed to 10 and 100 μM forskolin, a known direct activator of adenylyl cyclase that stimulated cAMP production to 2.99±0.4 and 8.33±0.5 nmol/mg protein, respectively ($P < 0.0001$, n = 4). Fig. 6 shows the expected exendin-(9–39) blockade of this response. The specific antagonist inhibited GLP-1–stimulated cAMP production in a concentration-dependent manner with a significant effect of dose vs. response [F(3,54) = 65.155; $P <$ 0.0001]. 33% and 87% reductions of GLP-1–stimulated cAMP production were achieved with 10 nM and 100 nM exendin-(9–39), respectively ($P < 0.0001$, n = 4). GLP-1 at concentrations up to 5 μM had no effect on intracellular Ca^{2+} in GT_1-7 cell suspensions in conditions where clear changes were observed in the presence of K^+ (20 mM) or histamine (100 μM; results not shown).

Effect of GLP-1 on LHRH release from whole hypothalami. Fig. 7 shows the results of an in vitro study performed to determine whether GLP-1 could stimulate LHRH release from intact male rat hypothalamic tissue. One supraphysiological dose of GLP-1 (1 μM) was administered to the hypothalami, and a significant increase in LHRH release was observed when compared with the basal value. Basal production was 0.3±0.07 fmol/mg wet wt/h, and GLP-1 increased this to 220% of basal (0.66±0.12 fmol/mg wet wt/h; $P < 0.04$; n = 12). A K^+ (56 mM) challenge significantly increased LHRH release from the hypothalami to 0.95±0.2 fmol/mg wet wt/h ($P < 0.03$; n =

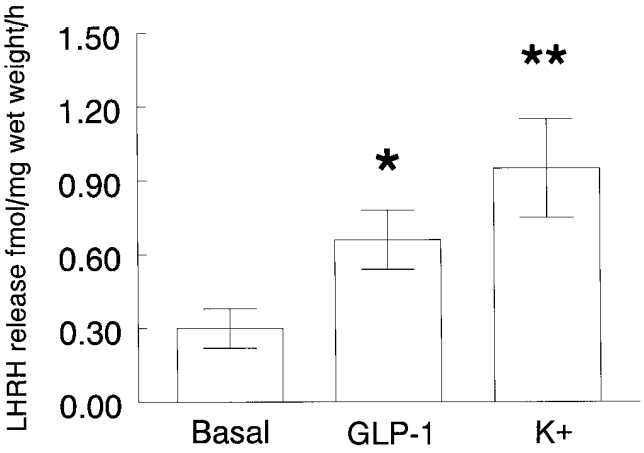

Figure 7. GLP-1 stimulation of basal LHRH release from isolated male rat hypothalami. LHRH levels were measured after a 60-min static incubation with GLP-1 (1 μM), and the viability of the tissue was verified by exposure to 56 mM KCl. (*$P < 0.05$ GLP-1 vs. control; **$P < 0.04$ KCl vs. control; n = 12).

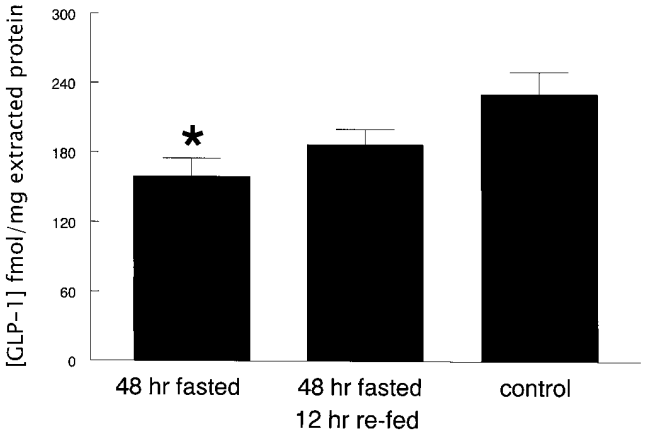

Figure 8. Effect of a 48-h fast and subsequent 12-h refeeding on hypothalamic GLP-1 content in male rats. Three groups of rats were fasted for 48 h, refed 12 h after a 48-h fast, or fed ad lib (control). Hypothalamic GLP-1 content was measured by RIA and expressed as fmol per mg extracted protein. (*$P < 0.01$ vs. control; $n = 15$).

12), suggesting that the tissue was able to respond to depolarization, and was therefore still viable.

Effect of intracerebroventricular injection of GLP-1 on LH release. The effect of intracerebroventricular injection of GLP-1 on the plasma LH of male rats in pilot studies (data not shown) indicated that GLP-1–stimulated LH release peaked at 5 min. GLP-1 (10 μg, 3 nmol) produced a significant increase in plasma LH levels 5 min after intracerebroventricular administration when compared with saline-injected controls (GLP-1: 1.09±0.11 ng/ml vs. saline: 0.69±0.06 ng/ml; $P < 0.005$). In addition, GLP-1 had no effect on plasma follicle-stimulating hormone, thyrotropin, or prolactin levels at this time point (data not shown).

Effect of fasting on GLP-1 content in the rat hypothalamus. Fig. 8 shows the results of a study performed to measure GLP-1 peptide content in male rat hypothalami in different states of fasting. It was clear from the study that nutritional balance appeared to affect hypothalamic GLP-1 levels [$F(2,42) = 5.13$; $P < 0.01$]. Hypothalamic GLP-1 levels in control rats that had ad libitum access to food were 230.4±18.9 fmol/mg extracted protein. After 48 h of fasting there was a significant decrease in GLP-1 content to 159.1±15.6 fmol/mg extracted protein ($P < 0.01$, $n = 15$). 48-h fasting followed by a 12-h refeed resulted in GLP-1 levels of 186.5±13.3 fmol/mg extracted protein. Although this group was not significantly different from either the control or the 48-h fasted group, there is indication of a trend that hypothalamic GLP-1 levels are increasing to normal upon refeeding.

Discussion

The GT_1-7 cells exhibit many of the known physiological characteristics of LHRH neurons in situ (30 and references therein); they thus provide a valuable model in which to study the mechanisms controlling release of the hypothalamic peptide. We have shown that GLP-1 causes a concentration-dependent increase in LHRH release from these cells ($ED_{50} = 0.28$ nM), and have also demonstrated the presence of specific GLP-1 binding sites in membrane homogenates. The sites meet the accepted criteria for a ligand–receptor interaction, being time-, temperature-, and protein content–dependent while displaying high affinity, saturability, and specificity. The ligand specificity exhibited by this GLP-1 binding site shows similarities with other GLP-1 receptors; i.e., the exendin peptides (31–33) bind with high affinity, and there is little cross-reactivity with other peptides belonging to the glucagon/secretin family. Analysis of the binding data identified a single high-affinity binding site ($K_d = 0.07$ nM; $B_{max} = 160$ fmol/mg protein) in the GT_1-7 cell membranes, a result analogous with that obtained for other GLP-1 binding sites (8, 34, 35).

To date, GLP-1 receptors in the brain and elsewhere have been shown to mediate an increase in cAMP levels via G-protein–coupled activation of adenylyl cyclase (9). Our results with the GT_1-7 cells are consistent with these findings. The threshold concentration of GLP-1 required to produce a significant rise in cAMP formation was equal to the threshold concentration required to significantly stimulate LHRH release from the cells, implying that receptor function is linked with the actions of GLP-1 on cAMP accumulation. In addition, the specific GLP-1 receptor antagonist, exendin-(9–39), inhibited this effect of GLP-1 in a concentration-dependent manner as previously shown in parietal (36) and α-TSH cells (19).

GLP-1 did not increase intracellular Ca^{2+} in GT_1-7 cells, even at 5 μM. This result contrasts with the reports of increased intracellular Ca^{2+} and activation of phospholipase C in COS-7 cells transfected with the cloned GLP-1 receptor. This result is unexpected as the GT_1-7 cell receptor appears to share very similar properties with the cloned GLP-1 receptor (37–39) in other respects such as binding of exendins, antagonistic effects of exendin-(9–39), and activation of adenylyl cyclase. Possible explanations may be that coupling to phospholipase C is cell-specific, or that overexpression in COS-7 cells led to coupling to G-proteins not normally associated with the GLP-1 receptor, although the high potency of the effect ($EC_{50} = 2$ nM) would argue against this idea. The lack of effect of GLP-1 on intracellular Ca^{2+} does not exclude other mechanisms, and we cannot prove categorically from this data that all effects of GLP-1 in these cells are mediated by cAMP.

Evidence presented in this paper leads us to propose that GLP-1 could be a factor involved in control of the hypothalamic-pituitary-gonadal axis. First, our in vitro studies demonstrate a clear ability of the peptide to stimulate release of LHRH both from an immortalized LHRH-secreting cell line (GT_1-7; reference 22) and from freshly removed rat hypothalamic tissue. We also show that the responses in the cell line are mediated via a specific GLP-1 receptor. Second, we show for the first time that GLP-1 stimulates release of LH in the male rat when injected into the third ventricle. Third, our previous in vitro studies indicate that GLP-1 does not act at the pituitary level to elicit LH release directly (19). Fourth, GLP-1 immunoreactive nerve fibres have been identified throughout the hypothalamus (4, 7, 40), including the rostral portion that also contains LHRH neurons and axon terminals (41). Moreover, GLP-1 receptor mRNA has been localized in the arcuate nucleus and medial preoptic area (14, 15); regions that are rich in LHRH neurons (42). Also of interest in this respect are recent observations demonstrating that female mice with a targeted disruption of the GLP-1 receptor gene exhibit delayed puberty (N.J. Maclusky, J. Kim, L. Scrocchi, and D. Drucker, personal communication). Taken together, these findings sug-

gest that GLP-1, like several other hypothalamic neuropeptides (43), plays a role as a neuromodulator of reproductive function.

Fertility in mammals requires adequate nutrition and fuel reserves (44). Energy restriction or high energy expenditure results in a failure of the hypothalamo-pituitary-gonadal axis, for example, in eating disorders (anorexia; reference 45), wasting diseases (insulin-dependent diabetes mellitus; reference 46), and high-performance athletes (47). Even short-term changes in food intake can disrupt reproduction in rats (48) and humans (49). A rapid reduction in pituitary LH and gonadal steroid secretion is seen with rapid reinstatement if food consumption is permitted (20). Experimental evidence suggests that the disruption may be in part attributable to perturbations in LHRH secretion (20). The effect of nutritional status on reproduction is thought to reflect the action of metabolic signals that serve as CNS indicators of metabolic state (50). Neuropeptide Y and galanin, for example, are neuropeptides that have been shown to modulate both food intake and the reproductive axis (20). Another potential messenger molecule that may play a role in manifesting reproductive disturbances during periods of energy imbalance is leptin, a hormone secreted from adipose tissue that has been shown to serve as a measure of body adiposity (51). Ob/ob mice are deficient in leptin, and show infertility that can be reversed by administration of leptin (21, 51). Furthermore, a recent study showed that leptin stimulated LHRH release from adult male rat hypothalamic explants in vitro and released LH in female rats in vivo (52).

Studies have defined a role for GLP-1 as a CNS satiety factor (10, 18, 53). It is possible that this peptide also acts as a messenger molecule between the neural processes that regulate reproduction, and those that maintain energy homeostasis. Perhaps after a short-term negative balance in energy, GLP-1, which is released in the brain after food intake, aids reinstatement of LH secretion. This hypothesis prompted us to investigate GLP-1 levels in the hypothalamus in a state of starvation or negative energy balance that would disrupt the reproductive axis. Indeed, we have shown here that after 48 h of fasting, rat hypothalamic GLP-1 content is significantly reduced, and upon refeeding a trend to return to normal levels was observed. This finding supports a possible physiological role for GLP-1 as a metabolic signal to the reproductive system. Hypothalamic GLP-1 levels are significantly low in conditions that would result in a rapid reduction in pituitary LH. Upon refeeding, the levels of GLP-1 increase, perhaps aiding reinstatement of LH secretion that has previously been observed (20). The findings presented here imply that GLP-1 may stimulate LH release, at least in part by activating of LHRH neurons. However, the mechanisms of action of this neuropeptide, either alone or in combination with other systems, especially neuropeptide Y and leptin, are at present not fully understood. It would be of great interest to examine the effect of GLP-1 on the more complex reproductive system of the female rat.

Acknowledgments

We would particularly wish to thank Professor Pam Mellon for supplying us with the GT_1-7 cells. The peptides used in this study were synthesized by Dr. Peter Byfield and Emma Stanton. We are also very grateful to Ms. M. Turton, Dr. M. Rossi, and Mr. S. Abusnana for help with the intracerebroventricular experiments.

S.A. Beak is funded by the British Diabetic Association, M.M. Heath is funded by the Medical Research Council (United Kingdom), and C.J. Small is funded by the Wellcome Foundation.

References

1. Mojsov, S., G. Heinrich, I.B. Wilson, M. Ravazzola, L. Orci, and J.F. Habener. 1986. Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. *J. Biol. Chem.* 261:11880–11889.

2. Yoshimoto, S., M. Hirota, C. Ohboshi, and K. Shima. 1989. Identification of glucagon-like peptide-1(7–36) amide in rat brain. *Ann. Clin. Biochem.* 26: 169–171.

3. Kreymann, B., S. Kanse, M.A. Ghatei, and S.R. Bloom. 1988. Glucagon-like peptide-1 7–36 amide, a new brain-gut hormone and its effect on pancreatic endocrine function in man. *Biomed. Res.* 9:207–211.

4. Kreymann, B., M.A. Ghatei, P. Burnet, G. Williams, S. Kanse, A.R. Diani, and S.R. Bloom. 1989. Characterization of glucagon-like peptide-1-(7–36)amide in the hypothalamus. *Brain Res.* 502:325–331.

5. Kreymann, B., Y. Yiangou, S. Kanse, G. Williams, M.A. Ghatei, and S.R. Bloom. 1988. Isolation and characterization of GLP-1 7–36 amide from rat intestine. Elevated levels in diabetic rats. *FEBS Lett.* 242:167–170.

6. Jin, S.L., V.K. Han, J.G. Simmons, A.C. Towle, J.M. Lauder, and P.K. Lund. 1988. Distribution of glucagon-like peptide I (GLP-I), glucagon, and glicentin in the rat brain: an immunocytochemical study. *J. Comp. Neurol.* 271: 519–532.

7. Shimizu, I., M. Hirota, C. Ohboshi, and K. Shima. 1987. Identification and localization of glucagon-like peptide 1 and its receptor in rat brain. *Endocrinology.* 121:1076–1082.

8. Kanse, S.M., B. Kreymann, M.A. Ghatei, and S.R. Bloom. 1988. Identification and characterization of glucagon-like peptide-1 7-36 amide-binding sites in the rat brain and lung. *FEBS Lett.* 241:209–212.

9. Fehmann, H.C., B. Goke, and R. Goke. 1995. Cell and molecular biology of the incretin hormones glucagon-like peptide-I and glucose-dependent insulin releasing polypeptide. *Endocr. Rev.* 16:390–408.

10. Turton, M.D., D. O'Shea, I. Gunn, S.A. Beak, C.M.B. Edwards, K. Meeran, S.J. Choi, G.M. Taylor, M.M. Heath, P.D. Lambert, et al. 1996. A role for glucagon-like peptide-1 in the central regulation of feeding. *Nature.* 379:69–72.

11. Goke, R., P.J. Larsen, J.D. Mikkelsen, and S.P. Sheikh. 1995. Identification of specific binding sites for glucagon-like peptide-1 on the posterior lobe of the rat pituitary. *Neuroendocrinology.* 62:130–134.

12. Calvo, J.C., B. Yusta, F. Mora, and E. Blazquez. 1995. Structural characterization by affinity cross-linking of glucagon-like peptide-1(7–36)amide receptor in rat brain. *J. Neurochem.* 64:299–306.

13. Thorens, B., A. Porret, L. Buhler, S.P. Deng, P. Morel, and C. Widmann. 1993. Cloning and functional expression of the human islet GLP-1 receptor. Demonstration that exendin-4 is an agonist and exendin- (9–39) an antagonist of the receptor. *Diabetes.* 42:1678–1682.

14. Alvarez, E., I. Roncero, J.A. Chowen, B. Thorens, and E. Blazquez. 1996. Expression of the glucagon-like peptide-1 receptor gene in rat brain. *J. Neurochem.* 66:920–927.

15. Shughrue, P.J., M.V. Lane, and I. Merchenthaler. 1996. Glucagon-like peptide-1 receptor (GLP1-R) mRNA in the rat hypothalamus. *Endocrinology.* 137:5159–5162.

16. Leibowitz, S.F. 1992. Neurochemical-neuroendocrine systems in the brain controlling macronutrient intake and metabolism. *Trends Neurosci.* 15: 491–497.

17. Schick, R.R., P. Zimmerman, T. Walde, V. Schusdziarra, and M. Classen. 1992. Glucagon-like peptide (GLP)-1-(7–36)amide: a central suppressor of food intake in fasted rats. *Gastroenterology.* 102(Abstr.):A756.

18. Tang-Christensen, M., P.J. Larsen, R. Goke, A. Fink-Jensen, D.S. Jessop, M. Moller, and S.P. Sheikh. 1996. Central administration of GLP-1(7–36)amide inhibits food and water intake in rats. *Am. J. Physiol.* 271:R848–R856.

19. Beak, S.A., C.J. Small, I. Ilovaiskaia, J.D. Hurley, M.A. Ghatei, S.R. Bloom, and D.M. Smith. 1996. Glucagon-like peptide-1 (GLP-1) releases thyrotropin (TSH): characterization of binding sites for GLP-1 on α-TSH cells. *Endocrinology.* 137:4130–4138.

20. Kalra, S.P., and P.S. Kalra. 1996. Nutritional infertility: the role of the interconnected hypothalamic neuropeptide Y-Galanin-Opioid network. *In* Frontiers in Neuroendocrinology. W.F. Ganong and L. Martini, editors. Raven Press, Ltd., New York. 371–401.

21. Barash, I.A., C.C. Cheung, D.S. Weigle, H. Ren, E.B. Kabigting, J.L. Kuijper, D.K. Clifton, and R.A. Steiner. 1996. Leptin is a metabolic signal to the reproductive system. *Endocrinology.* 137:3144–3147.

22. Mellon, P.L., J.J. Windle, P.C. Goldsmith, C.A. Padula, J.L. Roberts, and R.I. Weiner. 1990. Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron.* 5:1–10.

23. Kreymann, B., G. Williams, M.A. Ghatei, and S.R. Bloom. 1987. Glucagon-like peptide-1 7–36: a physiological incretin in man. *Lancet.* 2:1300–1304.

24. Gornall, A.G., C.J. Bardawill, and M.M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* 177:751.

25. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

26. Hunter, W.H., and F.C. Greenwood. 1962. Preparation of Iodine-131 labeled human growth hormone of high specific activity. *Nature.* 194:495–496.

27. Zamani, M., and D. Bristow. 1996. The histamine H1 receptor in GT1-7 neuronal cells is regulated by calcium influx and KN-62, a putative inhibitor of calcium/calmodulin protein kinase II. *Br. J. Pharmacol.* 118:1119–1126.

28. Morgan, D.G., P.D. Lambert, D.M. Smith, J.P.H. Wilding, and S.R. Bloom. 1996. Reduced NPY induced feeding in diabetic but not steroid-treated rats: lack of evidence for changes in receptor number or affinity. *J. Neuroendocrinol.* 8:283–290.

29. Leposavic, G., M. Dashwood, J. Ginsburg, and J.C. Buckingham. 1990. Peripubertal changes in the nature of the GnRH response to alpha-adrenoceptor stimulation in vitro and their modulation by testosterone. *Neuroendocrinology.* 52:82–89.

30. Besecke, L.M., A.M. Wolfe, M.E. Pierce, J.S. Takahashi, and J.E. Levine. 1994. Neuropeptide Y stimulates luteinizing hormone-releasing hormone release from superfused hypothalamic GT_1-7 cells. *Endocrinology.* 135:1621–1627.

31. Eng, J., W.A. Kleinman, L. Singh, G. Singh, and J.P. Raufman. 1992. Isolation and characterization of exendin-4, an exendin-3 analogue, from Heloderma suspectum venom. Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas. *J. Biol. Chem.* 267:7402–7405.

32. Eng, J., and C. Eng. 1992. Exendin-3 and -4 are insulin secretagogues. *Regul. Pept.* 40:(Abstr.):42.

33. Goke, R., H.C. Fehmann, T. Linn, H. Schmidt, M. Krause, J. Eng, and B. Goke. 1993. Exendin-4 is a high potency agonist and truncated exendin-(9–39)- amide an antagonist at the glucagon-like peptide 1-(7–36)-amide receptor of insulin-secreting beta-cells. *J. Biol. Chem.* 268:19650–19655.

34. Uttenthal, L.O., and E. Blazquez. 1990. Characterization of high-affinity receptors for truncated glucagon-like peptide-1 in rat gastric glands. *FEBS Lett.* 262:139–141.

35. Goke, R., and J.M. Conlon. 1988. Receptors for glucagon-like peptide-1(7–36) amide on rat insulinoma-derived cells. *J. Endocrinol.* 116:357–362.

36. Schepp, W., J. Schmidtler, T. Riedel, K. Dehne, V. Schusdziarra, J.J. Holst, J. Eng, J.P. Raufman, and M. Classen. 1994. Exendin-4 and exendin-(9–39)NH2: agonist and antagonist, respectively, at the rat parietal cell receptor for glucagon-like peptide-1-(7–36)NH2. *Eur. J. Pharmacol.* 269:183–191.

37. Wheeler, M.B., M. Lu, J.S. Dillon, X.H. Leng, C. Chen, and A.E. Boyd III. 1993. Functional expression of the rat glucagon-like peptide-I receptor, evidence for coupling to both adenylyl cyclase and phospholipase-C. *Endocrinology.* 133:57–62.

38. Widmann, C., E. Burki, W. Dolci, and B. Thorens. 1994. Signal transduction by the cloned glucagon-like peptide-1 receptor: comparison with signaling by the endogenous receptors of beta cell lines. *Mol.Pharmacol.* 45:1029–1035.

39. van Eyll, B., B. Lankat Buttgereit, H.P. Bode, R. Goke, and B. Goke. 1994. Signal transduction of the GLP-1-receptor cloned from a human insulinoma. *FEBS Lett.* 348:7–13.

40. Larsen, P.J., M. Tang-Christensen, J.J. Holst, and C. Orskov. 1997. Distribution of glucagon-like peptide 1 and other preproglucagon derived peptides in the rat hypothalamus and brainstem. *Neuroscience.* 77:257–270.

41. Merchenthaler, I., T. Gorcs, G. Setalo, and P. Petrusz. 1984. Gonadotropin-releasing hormone (GnRH) neurons and pathways in the rat brain. *Cell Tissue Res.* 237:15–29.

42. Kalra, S.P., and W.R. Crowley. 1984. Norepinephrine-like effects of neuropeptide Y on LH release in the rat. *Life Sci.* 35:1173–1176.

43. Gros, L., B. Thorens, D. Bataille, and A. Kervran. 1993. Glucagon-like peptide-1-(7–36) amide, oxyntomodulin, and glucagon interact with a common receptor in a somatostatin-secreting cell line. *Endocrinology.* 133:631–638.

44. Van Der Spuy, Z. 1985. Nutrition and reproduction. *Clin. Obstet. Gynecol.* 12:579–604.

45. Stewart, D. 1992. Reproductive functions in eating disorders. *Ann. Med.* 24:287–291.

46. Griffin, M., S. South, V. Yankov, R.J. Booth, C. Asplin, J. Veldhuis, and W. Evans. 1994. Insulin-dependent diabetes mellitus and menstrual dysfunction. *Ann. Med.* 26:331–340.

47. Cumming, D.C. 1993. The effects of exercise and nutrition on menstrual cycle. *In* Biomedical and Demographic Determinants of Reproduction. R. Gray, H. Leridon, and A. Spira, editors. Clarendon Press, Oxford, United Kingdom.

48. Cameron, J., and C. Nosbish. 1991. Suppression of pulsatile luteinizing hormone and testosterone secretion during short term food restriction in the adult male rhesus monkey (*Macaca mulatta*). *Endocrinology.* 128:1532–1540.

49. Cagampang, F., K. Maeda, A. Yokoyama, and K. Ota. 1990. Effect of food deprivation on the pulsatile LH release in the cycling and ovariectomized female rat. *Horm. Metab. Res.* 22:269–272.

50. Schreihofer, D., J. Amico, and J. Cameron. 1993. Reversal of fasting-induced suppression of luteinizing hormone (LH) secretion in male rhesus monkeys by intragastric nutrient infusion: evidence for rapid stimulation of LH by nutritional signals. *Endocrinology.* 132:1890–1897.

51. Chebab, F., M. Lim, and R. Lu. 1996. Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat. Genet.* 12:318–320.

52. Yu, W.H., M. Kimura, A. Walczewska, S. Karanth, and S.M. McCann. 1997. Role of leptin in hypothalamic-pituitary function. *Proc. Natl. Acad. Sci. USA.* 94:1023–1028.

53. Lambert, P.D., J.P.H. Wilding, M.A. Ghatei, and S.R. Bloom. 1994. A role for GLP-1(7–36)amide in the central control of feeding behavior. *Digestion.* 78(Abstr.).